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PRINCIPAL INVESTIGATOR: Warren D. Shlomchik, M.D.

CONTRACTING ORGANIZATION: Yale University School of Medicine
New Haven, CT 06529

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14. ABSTRACT
Our objective is to understand the immunobiology underlying the differential sensitivity of chronic phase and blast crisis CML. Our data thus far support the hypothesis that GVL against mCP-CML can be mediated by redundant processes, and that impairment of an individual pathway is insufficient to prevent GVL. We hypothesize that GVL against BC-CML is less forgiving than that against CP-CML, and that multiple effector pathways must act in concert for effective GVL. In the last year we have: 1) determined that cognate interactions are required for CD4 and CD8-mediated GVL; 2) determined that host antigen presenting cells are required for both CD4 and CD8-mediated GVL; 3) determined that killing by either FasL or TNF-α is not required for CD4 or CD8-mediated GVL; 4) created B7H1-/- mBC-CML; 5) determined that B7H1 expressed on mBC-CML does not impede CD8-mediated GVL; 6) created TGF-β-/- mBC-CML; and 7) determined that effector memory CD4 cells can mediate GVL against mBC-CML.

15. SUBJECT TERMS
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I. Introduction.

At the completion of this final year of funding we have completed nearly all proposed tasks and in addition we have taken the work into several new directions. The goal of our studies was to understand the immunogenicity of blast crisis CML and to compare these data to what we have learned about the immunogenicity of chronic phase CML. We have employed murine models of chronic phase CML (mCP-CML) and blast crisis CML (mBC-CML) induced by retroviral introduction of bcr-abl or bcr-abl and the NUP98/HOXA9 fusion cDNA, respectively. We have defined how T cells are activated to kill BC-CML cells and by what mechanisms they perform the killing. We have nearly completed this data set, and in sum we believe that GVL against mCP-CML and mBC-CML share the same mechanisms, but that mBC-CML cells are intrinsically resistant to the induction of apoptosis. I have summarized our findings in the abstract pasted below, which was accepted for an oral presentation at the 2006 meeting of the American Society of Hematology.

WARREN D. SHLOMCHIK, MD, CATHERINE MATTE-MARTONE, D.G. GILLILAND, LIEPING CHEN,

Cancer Center and Section of Immunobiology, Yale University School of Medicine, New Haven, CT, USA

Donor T cells mediate a graft-versus-leukemia effect that is responsible for much of the efficacy of allogeneic hematopoietic stem cell transplantation (alloSCT) in treatment of hematologic malignancies. Chronic phase chronic myeloid leukemia (CP-CML) is the most GVL-sensitive neoplasm. Unfortunately, most other malignancies are relatively GVL-resistant. A striking example is blast crisis CML (BC-CML) which, although sharing its genetic etiology with CP-CML, is nearly refractory to alloimmune T cells. A detailed understanding of GVL-resistance has been hindered by the absence of GVL-sensitive and GVL-resistant murine leukemias that are similar to their human counterparts and are inducible on different mouse strains. In particular, generating gene-deficient leukemias is important for mechanistic experiments. To address these limitations, we have adopted murine models of CP-CML (mCP-CML) and BC-CML (mBC-CML) that share pathology and genetic etiology with their human counterparts. mCP-CML is generated by retroviral transduction of murine bone marrow (BM) with the bcr-abl fusion cDNA (p210), the defining genetic abnormality in human CP-CML. As is the case with human CP-CML, mCP-CML is extremely GVL-sensitive at least in part due to the redundant immune mechanisms sufficient for GVL (Matte et al, Blood 2004). mBC-CML is induced by the retroviral transduction of BM with both p210 and the fusion cDNA NUP98/HOXA9 (Dash, PNAS, 2002), a translocation found in human BC-CML and AML. Relative to mCP-CML, mBC-CML is GVL-resistant. In the MHC-matched C3H.SW→B6 (H-2^b) strain pairing, 30-40% of recipients of 4-6 million donor CD4 or CD8 cells die from mBC-CML. This dose is nearly 10-fold higher than required for a similar survival from mCP-CML, even though recipients of mBC-CML and no donor T cells die nearly a week later than recipients of only mCP-CML. Having established that mBC-CML is GVL-resistant, we investigated mechanisms of T cell killing and the roles of donor and recipient antigen presenting cells (APCs). Direct T cell:mBC-CML cognate interactions were required as $MHCI^-$ and $MHCII^-$ mBC-CML cells (generated in $\beta_2$microglobulin (β_2M) or IA^b β chain knockout (KO) BM) were completely insensitive to CD8 and CD4-mediated GVL, respectively. In contrast, neither CD8 nor CD4-mediated GVL was impaired against mBC-CML generated from TNF-receptor1/2 double KO or Fas^{lpr} BM. These are the same basic mechanisms of cytotoxicity we observed in GVL against mCP-CML. CD8-mediated GVL against mBC-CML required functional recipient APCs as we observed no GVL when recipients were $MHCI^-$ β_2M KOs. As was the case with GVL against mCP-CML (Matte, N.Med. 2004), donor APCs were not required as GVL was equivalent in recipients of wild type and β_2M KO C3H.SW donor BM. We observed no GVL in $MHCII^-$ recipients demonstrating that CD4-mediated GVL also requires functional recipient APCs. In sum, the basic rules of immunogenicity for GVL against mCP-CML and mBC-CML are similar, suggesting that other pathways are responsible for GVL-resistance. One possibility is differential sensitivity to TRAIL-mediated killing and we are currently generating TRAILR-deficient mBC-CML. Another candidate is PD-L1, a B7 family member that can suppress T cell responses. PD-L1 is highly expressed on mBC-CML relative to mCP-CML. We have already generated PD-L1-deficient mBC-CML and GVL experiments with it are underway.

II. Body.

A. Aim 1 tasks.

1. Establish murine blast crisis (mBC-CML).

a) Generate appropriate retrovirus.

b) Infect B6 progenitors and transplant into B6 mice

c) Phenotype leukemia

d) Secondary transplants

All of these were accomplished in the first year of funding.

2. Establish GVL against B6 mBC-CML

Accomplished during the first year of funding.

3. Determine the roles of CD4 and CD8 cells.

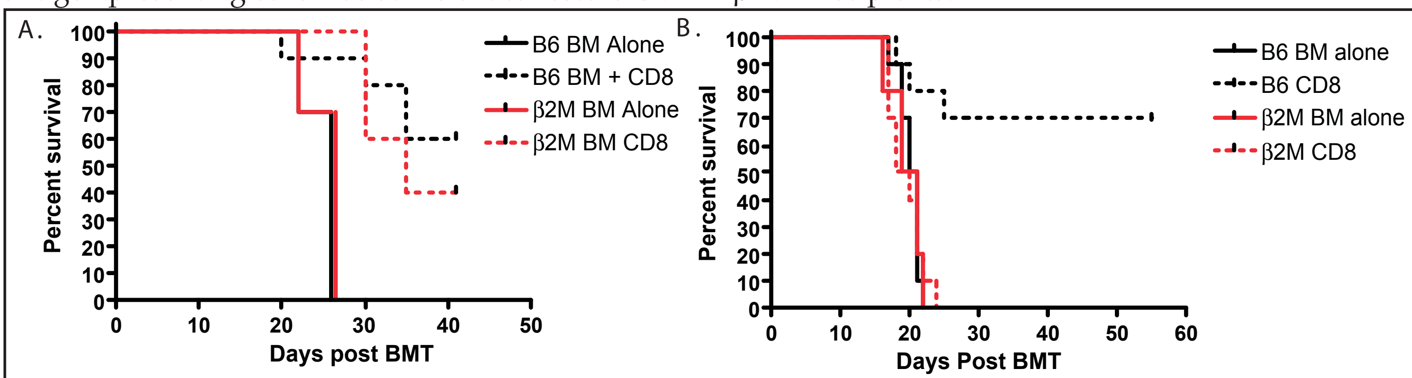
Accomplished during the first two years of funding.

4. Determine the minimum number of T cells

Performed during years 1 and 2. See prior T cell dose titration studies.

5. Determine the roles of recipient APCs in CD8 GVL.

In our year 2 update, we reported that recipient APCs were not required for CD8-mediated GVL. However, we included a caveat that the mBC-CML cells we used were not sort-purified and therefore could have been contaminated with recipient-type APCs which were sufficient to initiate GVL. Indeed this was the case as recipient β2M$^{-/-}$ B6 recipients, which lacked functional APCs, were completely resistant to CD8-mediated GVL against sort-purified mBC-CML cells (Figure 1). We also performed an experiment comparing CD8-mediated GVL in β2M$^{-/-}$ recipients against unsorted and sort-purified mBC-CML cells and GVL was intact against unsorted but not against sorted mBC-CML cells. Thus, a very small number of non-malignant recipient antigen presenting cells was sufficient to restore GVL in β2M$^{-/-}$ recipients.



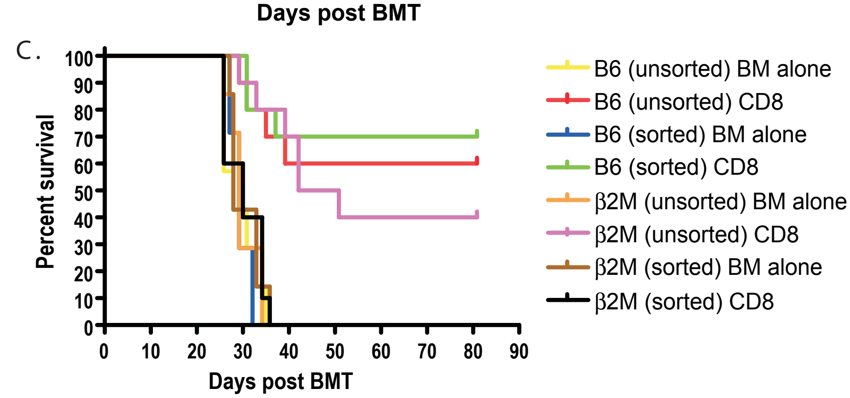


Figure 1. Recipient APCs are required for CD8-mediated GVHD. All experiments used the C3H. SW (H-2^b)→B6 (H-2^b) strain pairing. To impair host APCs we compared GVL in wt and β2M$^{-/-}$ recipients. We first examined GVL against unsorted B6 mBC-CML cells and found GVL to be equivalent in wt and β2M$^{-/-}$ recipients (A). To determine whether this surprising result was due to contaminating APCs in the mBC-CML cells, we repeated the experiment, this time with sort-purified B6 mBC-CML cells. With sorted BC-CML cells we observed no GVL in β2M$^{-/-}$ recipients (B; 1 of 2 similar experiments). Finally, to confirm that the different results in (A) and (B) were due to contaminating cells, we compared GVL in wt and β2M$^{-/-}$ recipients against sorted and unsorted mBC-CML cells, all in a single experiment. As shown in Fig. 1C, we observed no GVL in β2M$^{-/-}$ recipients with sorted mBC-CML cells; however, GVL in β2M$^{-/-}$ recipients was again intact when mBC-CML cells were unsorted and therefore contaminated with recipient type APCs.

6. Determine the roles of donor APCs in CD8 GVL.

To determine whether donor APCs are required for CD8-mediated GVL, irradiated B6 recipients were reconstituted with sort-purified B6 mBC-CML cells, either wild type or $\beta 2M^{-/-}$ C3H.SW BM, with or without C3H.SW CD8 cells. In these experiments, GVL was equivalent in CD8 recipients, regardless of whether the donor BM was $\beta 2M^{-/-}$ or wild type (Figure 2). Therefore, as was the case with GVL against mCP-CML, donor APCs were not required.

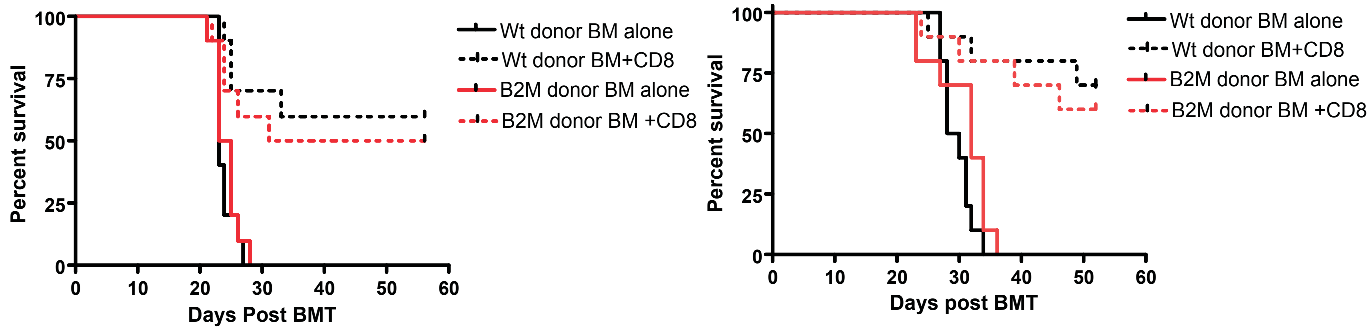
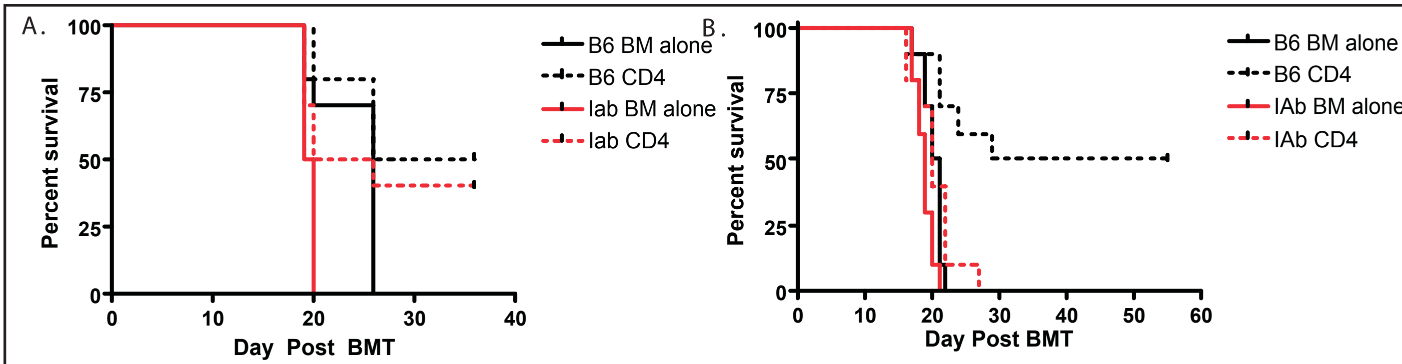


Figure 2. Donor APCs are not required for CD8-mediated GVL. To determine whether donor APCs are required for CD8-mediated GVL we compared GVL against mBC-CML using either wt or $\beta 2M^{-/-}$ donor BM. Shown are two repetitions. In each, recipients of C3H.SW $\beta 2M^{-/-}$ BM and wild type CD8 cells had similar survival as did recipients of wt CD8 cells. All deaths were due to mBC-CML.

7. Determine roles of recipient APCs, CD4 GVL.

In our year 2 update we reported that recipient APCs were not required for CD4-mediated GVL. We again raised the caveat that this could be the case due to contaminating recipient APCs in the unsorted mBC-CML cells. This was the case as B6 $IA^b\beta^{-/-}$ ($MHCII^-$) mice were resistant to CD4-mediated GVL against sort-purified mBC-CML cells (Figure 3).



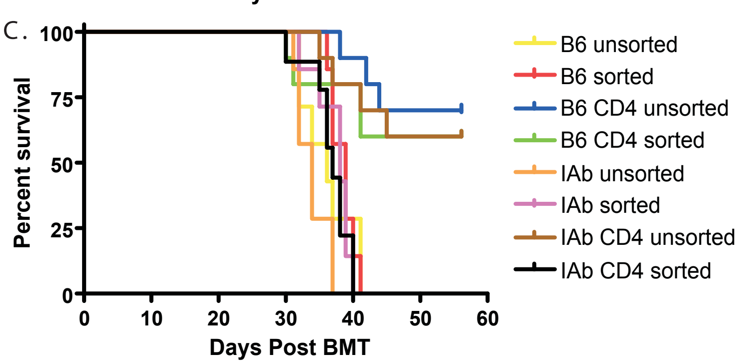


Figure 3. Host APCs are required for CD4-mediated GVL. All experiments were in the 129/J ($H\text{-}2^b$)→B6 ($H\text{-}2^b$) strain pairing. To impair the ability of recipient APCs to activate donor CD4 cells, we compared GVL in wt B6 and $IA^b\beta^{-/-}$ B6 recipients. In an initial experiment using unsorted B6 mBC-CML cells we observed similar GVL in wt and $IA^b\beta^{-/-}$ recipients (A). We repeated the experiment using sort-purified mBC-CML cells and found that GVL was absent in $IA^b\beta^{-/-}$ recipients of wild type CD4 cells (B). Finally, to confirm that the different results in (A) and (B) were due to contaminating cells, we compared GVL in wt and $IA^b\beta^{-/-}$ recipients against sorted and unsorted mBC-CML cells, all in a single experiment. As shown in Fig. 3C, we observed no GVL in $IA^b\beta^{-/-}$ recipients with sorted mBC-CML cells; however, GVL in $IA^b\beta^{-/-}$ recipients was again intact when mBC-CML cells were unsorted and therefore contaminated with recipient type APCs.

8. Induce mBC-CML in $MHCI^-$, $MHCII^-$ and $B71/B72^{-/-}$ mice. This task was accomplished in year 2.

9. Determine the role of leukemia antigen presentation. We initially proposed determining whether

leukemia APC function is sufficient and whether it is necessary. To determine whether it is sufficient, we utilized the B6.C→BALB/c strain pairing in which we have published that if both the recipient and donor are B71/B72$^{-/-}$, CD4-mediated GVHD is completely prevented [1]. To determine whether mBC-CML antigen presentation alone is sufficient we have performed GVL experiments in which the donor and host were B71/B72$^{-/-}$ and the leukemia is wild type. Thus, the only cells capable of priming GVL would be BALB/c mBC-CML cells. However, in two separate experiments mice that received only BALB/c mBC-CML and no T cells failed to develop leukemia. After several pilot experiments with a number of frozen vials of BALB/c mBC-CML cells, we determined that we must have frozen aliquots that are no longer leukemogenic. We have now nearly completed recloning the BALB/c mBC-CML cells from cells generated from the initial retroviral transduction and when these cells are validated we will repeat this experiment. We made B6 B71/B72$^{-/-}$ mBC-CML cells and we will use these shortly to determine if mBC-CML APC function is necessary for CD4-mediated GVL using the C3H.SW→B6 strain pairing.

B. Aim 2 Tasks.

1. Establish mBC-CML in β2M$^{-/-}$ and MHCII$^{-/-}$ (IA$^{b-/-}$) mice.

This was accomplished in year 2.

2. Test GVL against MHCI and MHCII deficient mBC-CML.

Determine if cognate interactions between effector T cells and leukemia targets is required *This was partially accomplished in year 2 and we report on the complete results here.* β2M$^{-/-}$ and therefore MHCI deficient mBC-CML was completely resistant to CD8-mediated GVL (Fig. 4). Similarly, IA^{b}β$^{-/-}$ and therefore MHCII^{-} mBC-CML was completely resistant to CD4-mediated GVL(Fig. 5).

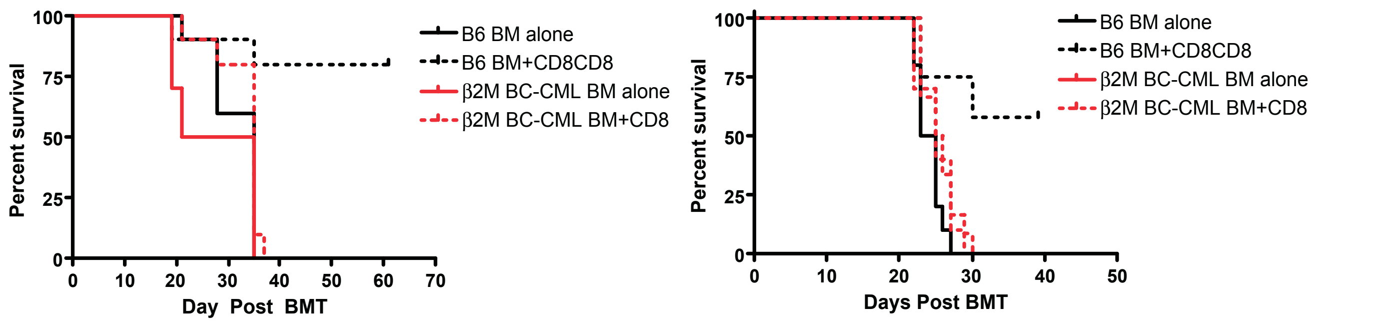


Figure 4. Cognate interactions between donor T cells and mBC-CML cells are required for CD8-mediated GVL. To determine whether cognate interactions are required for CD8-mediated GVL we compared GVL against B6 wt and B6 β2M$^{-/-}$ mBC-CML cells using the C3H.SW→B6 strain pairing. Because NK cells could reject β2M$^{-/-}$ mBC-CML cells, *all* mice were treated with anti-NK1.1 to deplete NK cells. In two repetitions (left and right panels), β2M$^{-/-}$ mBC-CML cells were completely resistant to CD8-mediated GVL.

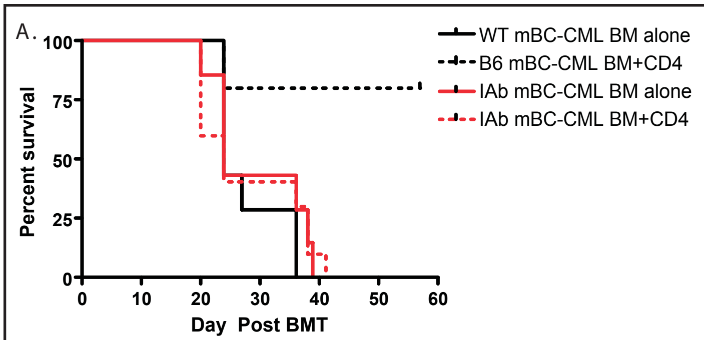


Figure 5. Cognate interactions between donor T cells and mBC-CML cells are required for CD4-mediated GVL. To determine whether cognate interactions are required for CD4-mediated GVL we compared GVL against B6 wt and B6 IA^{b}β$^{-/-}$ mBC-CML using the C3H.SW→B6 strain pairing. As we have previously reported for GVL against mCP-CML, IA^{b}β$^{-/-}$ mBC-CML cells were completely resistant to CD4-mediated GVL.

3. Establish mBC-CML in TNFR1/TNFR2$^{-/-}$, betac$^{-/-}$, Fas$^{-/-}$, IFN-γ receptor $^{-/-}$ mice.

We established TNFR1/TNFR2 and Fas^{lpr} mBC-CML cells in year 2. While we initially planned on making betac$^{-/-}$ and IFN-γ $^{-/-}$ mice, that cognate interactions were required for GVL made it less likely that these mechanisms would be key for GVL. We therefore have focused on other cytolytic mechanisms that require cognate interactions. Another important mechanism whereby T cells can kill targets is via the TRAIL receptor (TNF-related apoptosis inducing ligand receptor). We obtained TRAILR$^{-/-}$ mice from Astor Winoto and have made TRAILR$^{-/-}$ TRAILR$^{+/+}$ mBC-CML (the latter from littermate controls). Fortunately in the mouse there is only

one TRAILR and thus these cells should be completely resistant to TRAIL-mediated killing. These cells are currently being cloned in vivo. We have now also crossed TRAILR$^{-/-}$ mice to Fas^{lpr} mice. Thus mBC-CML cells generated from these mice will be resistant to both FasL and TRAIL mediated killing. We anticipate making this mBC-CML as soon as are colony is further expanded. Finally, we have obtained perforin$^{-/-}$ mice to use as T cell donors.

4. Test GVL against gene deficient leukemias in B.3. We have tested CD4 and CD8-mediated GVL against TNFR1/R2$^{-/-}$ (Fig. 6) and Fas^{lpr} mBC-CML (Fig. 7) and in both cases GVL was equivalent to that against wt mBC-CML. Therefore, individually impaired GVL against mBC-CML does not require killing by either FasL or TNF-α.

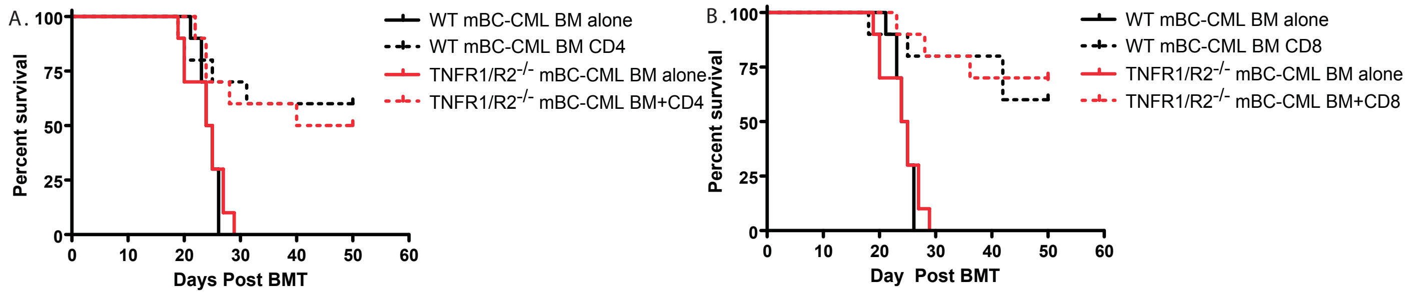


Figure 6. Killing via TNF-α is not required for CD4 or CD8-mediated GVL. To determine whether killing by TNF-α was required we compared GVL against B6 wt or B6 TNFR1/R2$^{-/-}$ mBC-CML cells. Neither CD4-mediated (A) nor CD8-mediated (B) GVL required killing by TNF-α as GVL was equivalent against wt and TNFR1/R2$^{-/-}$ mBC-CML cells.

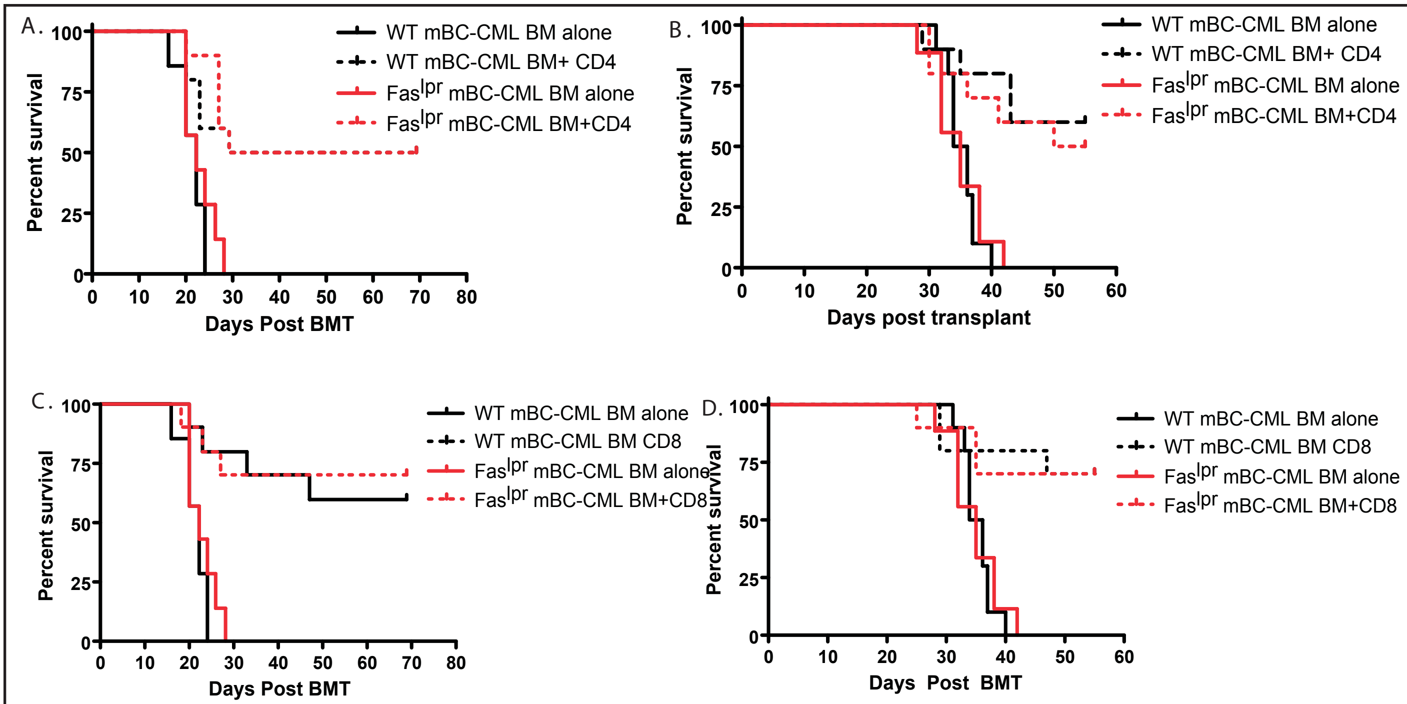


Figure 7. Killing via FasL is not required for CD4 or CD8-mediated GVL. To determine whether killing by FasL was required we compared GVL against B6 wt or B6 Fas^{lpr} mBC-CML cells. Neither CD4-mediated (A and B, 2 repetitions) nor CD8-mediated (C and D, 2 repetitions) GVL required killing by FasL as GVL was equivalent against wt and Faslpr mBC-CML cells.

C. Additional Data and New Plans.

1. The potential role of B7H1 and B7DC in rendering mBC-CML cells relatively resistant. In our year 2 update we reported that mBC-CML expresses high levels of B7-H1, which is a B7-family member that is a ligand for the receptor PD-1, expressed on activated T cells. PD-1 inhibits T cell activation and function and has been implicated in resistance to T cell-mediated anti-tumor responses. We have since generated mBC-CML in bone marrow from B6 B7H1$^{-/-}$ mice (obtained from Leiping Chen) (Figure 8A). We then compared CD8-mediated GVL against wild type and B7H1$^{-/-}$ mBC-CML. However, GVL was equivalent when it

was mediated by either 4x10⁶ of 10⁶ donor CD8 cells (Fig. 8B). We were somewhat surprised by this result. However, we had previously noted that mBC-CML also express B7DC, which is also a ligand for PD1. Thus we have also obtained B7DC$^{-/-}$ mice (from Drew Pardoll) and we have made mBC-CML cells from BM from these mice. However, we are now fortunate to have obtained mice doubly deficient in both B7H1 and B7DC (created by Arlene Sharpe and obtained from Bruce Blazar). These mice have just cleared quarantine and are breeding in our colony. When we create mBC-CML from bone marrow from B7H1/B7DC$^{-/-}$ mice we will completely prevent signaling via PD1.

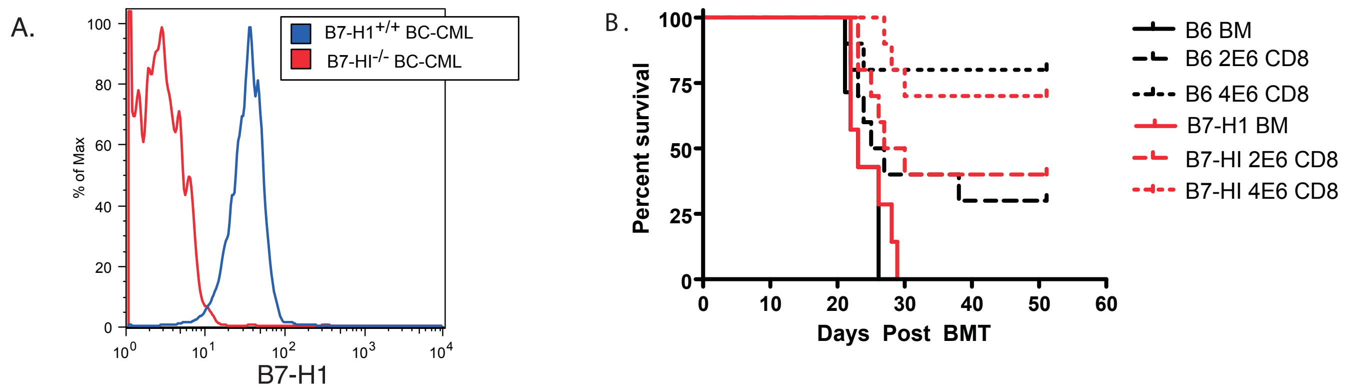


Figure 8. B7H1$^{-/-}$ mBC-CML is not more sensitive to CD8-mediated GVL. We created B7H1$^{-/-}$ mBC-CML from bone marrow from B7H1$^{-/-}$ mice backcrossed to B6. Shown in (A) is staining of B7H1$^{-/-}$ and B7H1$^{+/+}$ mBC-CML with anti-B7H1 (plot is gated on NGFR$^+$EGFP$^+$ cells). We tested whether B7H1$^{-/-}$ mBC-CML is more sensitive to GVL using the C3H.SW→B6 strain pairing. We compared a high (4x10⁶) and a low (2x10⁶) CD8 dose in order to bring out a difference (B). Nonetheless, wild type and B7H1$^{-/-}$ mBC-CML were equally sensitive to CD8-mediated GVL.

2. Generation of mBC-CML cells that do not express TGF-β or overexpress TGF-β. Another potential mechanism for GVL resistance could be leukemic expression of TGF-β. In collaboration with Richard Flavell we plan to test this hypothesis by creating leukemic cells that either overexpress TGF-β or do not express it at all. To do so we have created a retrovirus that expresses p210 and cre recombinase and another retrovirus that expresses p210 and TGF-β 1. We have already used the cre-expressing virus to create mBC-CML cells that do not express TGF-β1. We did this by infecting bone marrow from mice with one deleted TGF-β1 allele and one floxed TGF-B1 allele. As a control we infected BM from TGF-β1$^{+/-}$ mice. These leukemias have completed a second passage and have been sort-purified. They are currently being analyzed for successful deletion of the TGF-β1 allele and for TGF-β1 production. If we validate that we have indeed created TGF-β1 deficient mBC-CML and that wild type mBC-CML cells express TGF-β1, then we will use these leukemias in GVL experiments. We anticipate making mBC-CML that over-expresses TGF-β1 by early 2007.

3. Effector memory CD4 cells mediate GVL against mBC-CML without causing GVHD. My lab has been investigating the properties of effector memory T cells (T_{EM}) in allogeneic stem cell transplantation. We reported that T_{EM} do not cause GVHD, but engraft and transfer functional T cell memory [2]. We have also investigated whether T_{EM} mediate GVL, and in abstract form we have reported that CD4$^+$ T_{EM} mediate GVL

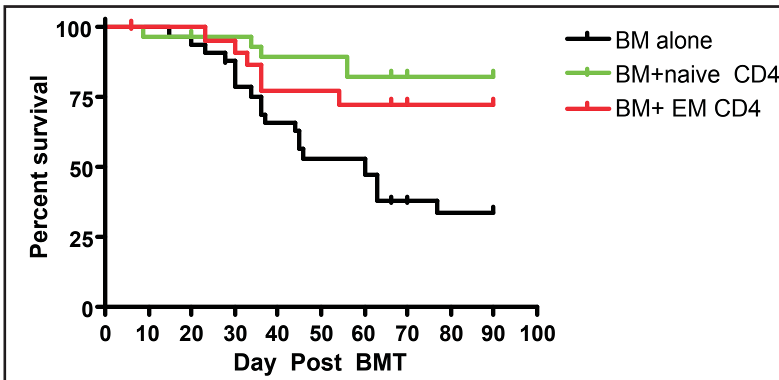


Figure 9. Effector memory CD4 cells can mediate GVL against mBC-CML cells. To determine whether effector memory CD4 cells (CD4$^+$ T_{EM}) could mediate GVL, B6 mice were irradiated and reconstituted with B6 mBC-CML cells, T cell depleted B6^{bm12} (MHCII-disparate) BM, with no T cells (BM alone), naïve (CD44$^-$CD62L$^+$) CD4 cells or T_{EM} CD4 cells (CD44$^+$CD62L$^-$). T_{EM} recipients had improved survival as compared to recipients of no T cells (P=0.0123). All deaths in BM alone and T_{EM} recipients were from mBC-CML. Deaths in recipients of naïve CD4 cells was from GVHD. Data is combined from 3 repetitions. For reasons we do not understand, in the B6^{bm12}→B6 strain pairing, all recipients of mBC-CML cells and no donor T cells did not die from mBC-CML. Nonetheless, the survival differences are highly significant and indicate that T_{EM} CD4 cells can mediate GVL against mBC-CML cells in this MHCII-disparate model system.

against mCP-CML in the MHCII-disparate B6^{bm12}→B6 strain pairing. We have also investigated whether T_{EM} mediate GVL against mBC-CML. To do so B6 mice were irradiated and reconstituted with 10^4 B6 mBC-CML cells, T cell depleted B6^{bm12} bone marrow, with no T cells, 10^6 CD4$^+$ T_{EM} or CD4$^+$ naïve T cells (T_N). Show in in Figure IX is data combined from 4 experiments. CD4$^+$ T_{EM} have a significant survival advantage over mice that received no T cells (Fig. 9). Thus CD4$^+$ T_{EM} mediate GVL and suggest that this could be an effective strategy for improving GVL in MHCII mismatched human transplants. I have already won a National Cancer Institute Rapid Access to Intervention Development award (RAID) to create a reagent to purify memory T cells for use in human allogeneic hematopoietic stem cell transplantation.

III. Key Results From Year 3.

A. Host APCs are required for CD8$^-$mediated GVL.

B. Donor APCs are not required for CD8-mediated GVL.

C. Host APCs are required for CD4-mediated GVL.

D. Killing via FasL is not required for CD4 or CD8-mediated GVL.

E. Killing via TNF-α is not required for CD4 or CD8-mediated GVL.

F. CD8-mediated GVL requires cognate interactions with mBC-CML targets.

G. CD4-mediated GVL requires cognate interactions with mBC-CML targets.

H. The absence of B7H1 mBC-CML cells does not enhance CD8-mediated GVL.

IV. Reportable outcomes. Please see the abstract at the beginning of this document. A manuscript that encompasses this data is currently being prepared.

V. Conclusions

In sum our data thus far lead us to believe that the basic rules of immunogenicity are shared in GVL against GVL-sensitive mCP-CML and GVL-resistant mBC-CML. That is antigen presentation requirements and T cell effector mechanisms are equivalent. In the next 12 months we should have further information on the requirements for perforin and TRAILR for both mCP-CML and mBC-CML. If these experiments are unrevealing we will begin searching for other potential explanations. We will continue to pursue the potential roles of PD1 ligands and TGF-β as described above. We have not yet tested the hypothesis that mBC-CML cells are resistant to GVL due to reduced expression of adhesion molecules essential for T cell recognition. We would address this by first characterizing adhesion molecule expression by flow cytometry. If we see notable examples of a molecule having higher expression in mCP-CML cells relative to mBC-CML cells, we could overexpress it in mBC-CML cells with retrovirus, or decrease its expression in mCP-CML cells by either infecting BM from mice genetically deficient in it (if available) or via an RNAi approach. We will also begin to test the hypothesis that mBC-CML cells are GVL-resistant due to anti-apoptotic mechanisms downstream of death receptors and granzyme-mediated induction of apoptosis. We are considering a gene expression profiling approach to try to discern differences between blast crisis cells and chronic phase cells. To do so we will want to compare clonogenic leukemia cells. mBC-CML cells are relatively homogeneous and we believe that a significant fraction of cells that we collect from spleen or BM are clonogenic. However, mCP-CML is more heterogeneous and identification of a clonogenic progenitor could be more difficult. We could also examine several candidate genes (bcl2, bclXL, etc.).

VI. References

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